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REVERSIBLE INHIBITION OF SODIUM AND POTASSIUM-DEPENDENT ADENOSINE TRIPHOSPHATASE BY THE PYRIDINE DERIVATIVE, AU-1421 DURING TURNOVER CYCLE

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Abstract—A novel pyridine derivative, (*Z*)-5-methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidonopyridine hydrochloride, AU-1421, was found to produce reversible inhibition of the dog kidney sodium and potassium ion-dependent adenosine triphosphatase [(Na,K)-ATPase] with I_{50} values of about 50 μM. The reversible inhibition was observed when the enzyme was added directly to the enzyme assay media in the presence of saturating concentrations of the enzyme ligands, Na^+, K^+, Mg^{2+} and ATP ("turnover conditions"). In the present study, we focused on the reversible inhibition without preincubation of the enzyme with AU-1421. This inhibition was competitive with respect to K^+. The K^+-pNPPase activity of the same preparation was also inhibited by AU-1421 with I_{50} values of about 90 μM, and this manner was also competitive with respect to K^+. ATP enhanced the AU-1421 inhibition of (Na,K)-ATPase, suggesting that AU-1421 also bound to the enzyme–substrate complex. AU-1421 inhibition of (Na,K)-ATPase was not antagonized by ouabain, suggesting the difference of the binding sites between AU-1421 and ouabain. It is therefore proposed that AU-1421 reversibly interacts at or near the K^+ site during turnover conditions.

A novel pyridine derivative, (*Z*)-5-methyl-2-[2-(1-naphthyl)ethenyl]-4-piperidinopyridine hydrochloride (AU-1421), is an effective inhibitor of gastric acid secretion *in vivo* and is a reversible K^+-competitive inhibitor of (H,K)-ATPase* *in vitro* [1]. In contrast, AU-1421 has access specific to the K^+ occlusion center of (Na,K)-ATPase under non-turnover conditions (in the absence of enzyme ligands, Na^+, K^+, Mg^{2+} and ATP), where relatively high temperature and preincubation time of the order of minutes were required for stable binding of AU-1421 [2]. This binding is apparently irreversible. In terms of enzymatic mechanisms, there are many features shared by phosphorylating transport ATPases, especially (Na,K)-ATPase and (H,K)-ATPase [3–5], and there is a 60% homology in the primary sequences of their catalytic subunits [5–8]. Despite these general similarities, the degree of K^+ occlusion in (H,K)-ATPase is less obvious than that in (Na,K)-ATPase [9, 10]. The difference of AU-1421 binding may be due to the different feature of K^+-access sites between both ATPases. In the light of these experiences, it was considered desirable to investigate the mechanism of action of AU-1421 with the K^+-activating kinetics of the (Na,K)-ATPase and the accompanying K^+-pNPPase (partial reaction of (Na,K)-ATPase) under turnover conditions (in the presence of enzyme ligands).

In this paper kinetic observations are described which show that AU-1421 produces reversible inhibition of (Na,K)-ATPase without preincubation of the enzyme with AU-1421. The inhibition was competitive with respect to K^+ activation in both of the Na,K-ATPase and the K^+-pNPPase.

MATERIALS AND METHODS

Measurement of (Na,K)-*ATPase activity*. Dog kidney (Na,K)-ATPase was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) (grade IV), and ATPase activity was tested at 37° in the following incubation medium, unless otherwise stated: 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM Tris · ATP, 50 mM Tris–HCl (pH 7.4) and 30 μg of enzyme protein in a final volume of 0.5 mL. (Na,K)-ATPase activity was calculated by subtracting the activity seen in the absence of stimulating cation (Na^+,K^+) from the total activity. The incubation time was 15 min. The reaction was started by the addition of enzyme and stopped by the addition of 1 mL of cold 12% (w/v) perchloric acid containing 3.6% (w/v) ammonium molybdate. Inorganic phosphate was determined according to Yoda and Hokin [11].

Measurement of K^+-*pNPPase activity*. K^+-pNPPase activity was tested at 37° in the following incubation medium, unless otherwise stated: 10 mM KCl, 3 mM $MgCl_2$, 3 mM di(Tris) · pNPP, 50 mM Tris–HCl (pH 7.4) and 30 μg of enzyme protein in a final volume of 0.5 mL. The other conditions were the same as the case of (Na,K)-ATPase activity.

Chemicals. The Tris salt of ATP (equine muscle)

* Abbreviations: (Na,K)-ATPase, sodium and potassium ion-dependent adenosine triphosphatase (EC 3.6.1.3.); (H,K)-ATPase, proton and potassium ion-dependent adenosine triphosphatase from gastric mucosa (EC 3.6.1.36); K^+-pNNPase, potassium ion-dependent *p*-nitrophenyl phosphatase (EC 9.6.1.7.); pNPP, *p*nitrophenyl phosphate, E_1, E_2, conformations of (Na,K)-ATPase; E(ion), an occluded conformation containing an ion.

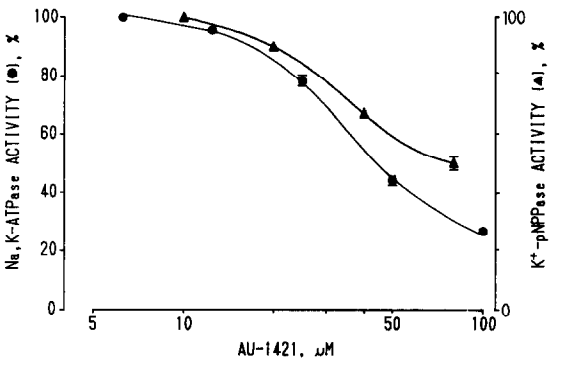

Fig. 1. Inhibition of (Na,K)-ATPase (●) and K^+-pNPPase (▲) activity by AU-1421. Standard incubation condition was used. Each point represents the mean of 3 experiments (N = 3).

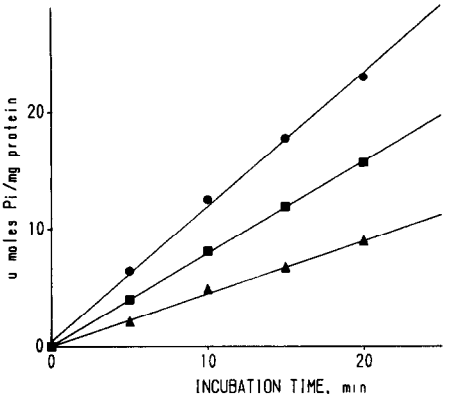

Fig. 2. Time course of inhibition of (Na,K)-ATPase activity in the absence (●) and in the presence of (■) 30 or (▲) 60 μM AU-1421. Standard incubation condition was used, except that incubation time varied from 5–20 min (N = 3).

was purchased from the Sigma Chemical Co. AU-1421 was synthesized at Banyu (Tokyo, Japan), and was dissolved in distilled water in all experiments. All other chemicals used were of analytical grade or the highest purity available.

Analysis of kinetic data. V_{max} values were estimated graphically from Lineweaver–Burk plots, and $K_{0.5}$ and Hill coefficients *(n)* were calculated from Hill plots, i.e. $\log(V/V_{max}-V)$ with respect to log[KCl or NaCl] with straight lines drawn by the method of least squares. I_{50} values were calculated from concentration–inhibition curves using a logit transformation. All values are expressed as the means ± SE.

RESULTS

Enzyme inhibitory effects of AU-1421

When AU-1421 was added directly to the enzyme assay media in the presence of saturating concentrations of the enzyme ligands, concentrations of AU-1421 required for 50% inhibition values (I_{50}) of (Na,K)-ATPase and K^+-pNPPase were 50 and 90 μM, respectively (Fig. 1). This inhibition was independent of incubation time, indicating that true equilibrium was readily attained (Fig. 2). Therefore, this behavior is suggestive of a reversible interaction between the enzyme and AU-1421.

Effects of Na^+ and K^+ concentrations

The effect of K^+ concentration on the AU-1421 inhibition of the (Na,K)-ATPase is shown as a representative double reciprocal plot in Fig. 3. AU-1421 is a simple competitive inhibitor of K^+ activation, as the extent of inhibition approaches zero as the K^+ concentration is increased. The apparent affinity of K^+ for the enzyme was decreased reflected by the observation that the concentration for half maximal activation ($K_{0.5}$) increased from 1.24 mM to 3.89 mM in the presence of 32 μM AU-1421. The apparent V_{max} was not affected. The degree of sigmoidicity measured as the slope of the Hill plot, an expression of the cooperative activation of this enzyme by K^+,

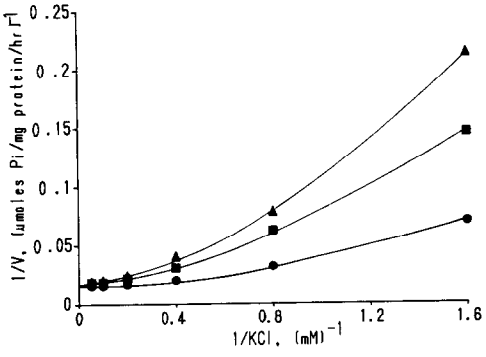

Fig. 3. Lineweaver–Burk plot of the effect of varying the K^+ concentration on the (Na,K)-ATPase activity in the absence (●) and in the presence of (■) 26 or (▲) 32 μM AU-1421. Standard incubation condition was used, except that KCl concentration varied from 0.625–20 mM (N = 3).

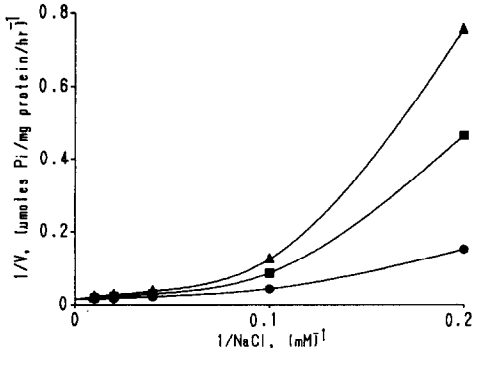

Fig. 4. Lineweaver–Burk plot of the effect of varying the Na^+ concentration on the (Na,K)-ATPase activity in the absence (●) and in the presence of (■) 24 or (▲) 33 μM AU-1421. Standard incubation condition was used, except that NaCl concentration varied from 5–100 mM (N = 3).

was decreased from 2.09 ± 0.06 (range N = 3) to 1.38 ± 0.06 (range N = 3) in the presence of 32 μM AU-1421 (plots not shown). The effect of Na^+ concentration on the inhibition of the (Na,K)-ATPase is shown in Fig. 4 as a representative Lineweaver–

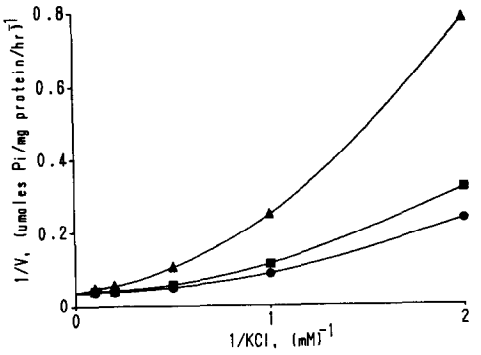

Fig. 5. Lineweaver–Burk plot of the effect of varying the K^+ concentration on the K^+-pNPPase activity in the absence (●) and in the presence of (■) 15 or (▲) 30 μM AU-1421. Standard incubation condition was used, except that KCl concentration varied from 0.5–10 mM (N = 3).

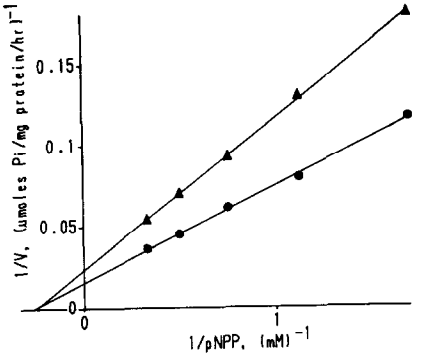

Fig. 7. Lineweaver–Burk plot of the effect of varying the pNPP concentration on the K^+-pNPPase activity in the absence (●) and in the presence of (▲) 35 μM AU-1421. Standard incubation condition was used, except that pNPP concentration varied from 0.59–3 mM (N = 3).

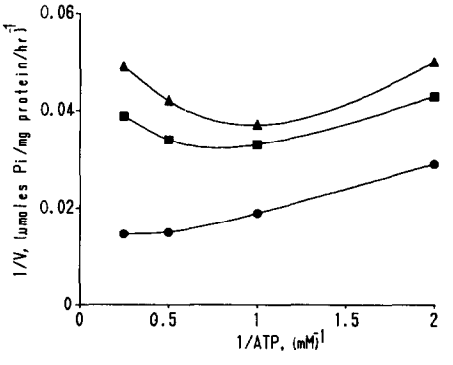

Fig. 6. Lineweaver–Burk plot of the effect of varying the ATP concentration on the (Na,K)-ATPase activity in the absence (●) and in the presence of (■) 36 or (▲) 48 μM AU-1421. Standard incubation condition was used, except that Tris–ATP concentration varied from 0.5–3 mM and 15 μg of enzyme was used to depress ATP depletion (N = 3).

Burk plot. AU-1421 inhibition was also competitive with respect to Na^+, without altering V_{max} and increasing the $K_{0.5}$, from 15.8 mM to 45.8 mM in the presence of 33 μM AU-1421. However, no change in cooperativity of activation was observed (plots not shown). The effect of varying K^+ concentration on the inhibition of the K^+-pNPPase was also investigated. The representative Lineweaver–Burk plot in Fig. 5 shows that AU-1421 inhibits the K^+-pNPPase competitive with respect to K^+, without altering V_{max} and increasing the $K_{0.5}$, from 1.16 mM to 2.91 mM in the presence of 30 μM AU-1421. The degree of cooperativity decreased very slightly, from 1.87 ± 0.08 (range N = 3) in the absence to 1.59 ± 0.10 (range N = 3) in the presence of 30 μM AU-1421 (plots not shown).

Effects of ATP and pNPP

The effects of AU-1421 on the kinetics of ATP hydrolysis are shown in Fig. 6 as a Lineweaver–Burk plot. AU-1421 caused an upward shift of the control line, decreasing $K_{0.5}$ and V_{max}. Inhibition of the (Na,K)-ATPase by 36 μM AU-1421 increased from 31%, at 0.5 mM ATP, to 60%, at 3 mM ATP. The effects of AU-1421 on the kinetics of pNPP hydrolysis are shown in Fig. 7 as a Lineweaver–Burk plot. AU-1421 inhibition was noncompetitive with respect to pNPP, without altering the $K_{0.5}$ (3.2 mM) and decreasing V_{max}.

Effects of ouabain on (Na,K)-*ATPase in the presence of AU-1421*

The interaction between AU-1421 and ouabain was examined, since ouabain also competes with K^+ in terms of inhibition of (Na,K)-ATPase activity. Ouabain and AU-1421 inhibited the (Na,K)-ATPase with I_{50}, 0.78 ± 0.04 (range N = 3) and 17.5 ± 0.71 (range N = 3), respectively. The effects of AU-1421 inhibition of the (Na,K)-ATPase were not antagonized by ouabain (Fig. 8). The I_{50} values for ouabain in the presence of AU-1421 and that for AU-1421 in the presence of ouabain were 0.72 ± 0.07 (range N = 3) and 16.5 ± 0.7 (range N = 3), respectively.

DISCUSSION

The inhibition of (H,K)-ATPase by AU-1421 has been reported to be both reversible and competitive with respect to the activating cation, K^+ [1]. In this study, the mechanism by which AU-1421 inhibits (Na,K)-ATPase is kinetically competitive with respect to K^+ under turnover conditions. This suggests that AU-1421 could act at the extracellular K^+ sites, since extracellular K^+ stimulates ATPase activity by increasing the rate of dephosphorylation of the phosphoenzyme (E_2P) intermediate. Actually AU-1421 prevented this dephosphorylation step as would be expected for a true K^+ site inhibitor (unpublished observation). In addition, AU-1421 showed a higher affinity for (Na,K)-ATPase in the inhibition of ATPase activity as compared with pNPPase. Since pNPP hydrolysis is not believed to occur via a phosphoenzyme intermediate [12], this might reflect a higher affinity of AU-1421 for the phosphorylated intermediate of the ATPase

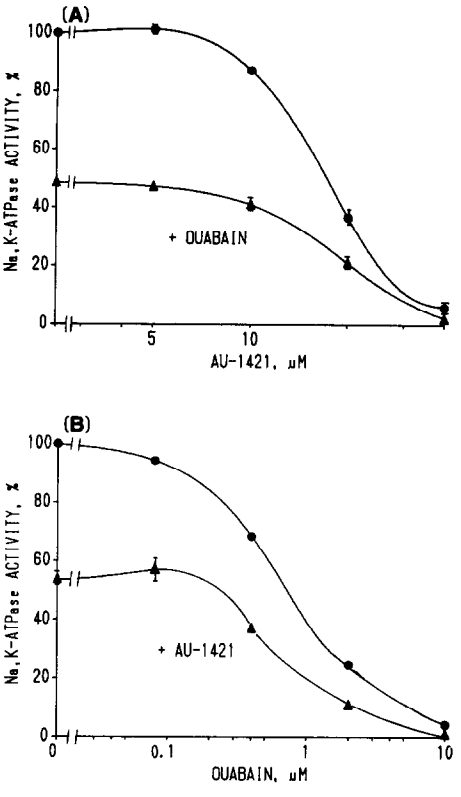

Fig. 8. (A) Effect of various concentrations of AU-1421 on the (Na,K)-ATPase activity in the absence (●) and in the presence (▲) of 0.8 μM ouabain. (B) Effect of various concentrations of ouabain on Na,K-ATPase activity in the absence (●) and in the presence of (▲) 15 μM AU-1421. Inhibitors were preincubated for 30 min at 37° prior to ATPase assay (N = 3).

reaction. Although competitive interaction between two ligands does not necessarily affirm a common site of action, the simplest hypothesis to explain these results is that both (H,K)-ATPase and (Na,K)-ATPase have at least in part common sequences in their sites of extracellular K^+-binding.

In the K^+ transport steps of the Na-pump cycle, K^+ become "occluded" within the pump molecule, inaccessible to both intracellular and extracellular media [13, 14]. Occluded K^+ are released into the intracellular media subsequent to the binding of ATP at a low affinity site of the enzyme, accompanying an E_1–E_2 conformational change in the enzyme. This step appears to be rate limiting for this enzyme [15]. Kinetic analysis shows that inhibition by AU-1421 is enhanced by ATP, suggesting that binding of ATP is followed by a conformational change, from $E_2(K)$ to E_1ATP, which greatly facilitates interaction of AU-1421 with (Na,K)-ATPase. Actually AU-1421 can bind effectively to dephosphorylated form, presumably from the intracellular side under non-turnover conditions, and binding of ATP at a low affinity site increase AU-1421 binding [2]. Therefore, the secondary binding route of AU-1421 may be a K^+-releasing pathway facing the intracellular side, which is open on the E_1 form of the enzyme.

AU-1421 inhibition of the K^+-pNPPase is competitive with respect to K^+. This result is consistent with the case of Na,K-ATPase. In addition, AU-1421 decreased the cooperative interaction of the K^+ sites of Na,K-ATPase and K^+-pNPPase. These results support the hypothesis that AU-1421 interacts at K^+ sites. However, K^+-pNPPase activity is stimulated by K^+ on the intracellular side of the membrane. This might reflect the interaction of AU-1421 with K^+ sites of dephosphorylated enzyme from the intracellular side.

Since AU-1421 competes with Na^+ in terms of inhibition of (Na,K)-ATPase activity, another possibility is that AU-1421 interacts with Na^+ site. In our previous experiments, AU-1421 did not compete with Na^+ at the high-affinity site [2]. Thus, AU-1421 may not bind directly at the Na^+ site. Probably, this result is due to the induction of mutually exclusive conformations of the enzyme, E_1Na or $E_2P \cdot I$. Further studies of the effects of AU-1421 on phosphoenzyme levels may help to resolve this issue.

When the enzyme was preincubated with AU-1421 in the absence of the enzyme ligands and then assayed in the presence of saturating levels of the ligands, the inhibition was apparently irreversible [2]. This inhibition was prevented, with simple competitive kinetics, by K^+ or its congeners [2]. The enzyme–inhibitor complex may form a very stable occluded form as enzyme–K^+ complex does, although AU-1421 binding requires relatively high temperature and a preincubation time of the order of minutes to be bound stable in the enzyme [2]. Since the K^+ occlusion center of (Na,K)-ATPase is thought to exist in the hydrophobic interior domain of the enzyme molecule [2, 16], these considerations suggest that the target site of AU-1421 is at or near the K^+ occlusion center during non-turnover conditions.

The interaction of ouabain with this enzyme is promoted by Na^+ and competitive with K^+ at low concentrations and mixed competitive and non-competitive at higher concentrations [17]. In contrast with ouabain inhibition, AU-1421 has been found to inhibit the (Na,K)-ATPase competitively with K^+ and Na^+. Further, the AU-1421 inhibition of (Na,K)-ATPase was not antagonized by ouabain, suggesting that the AU-1421 binding site is different from the ouabain binding site.

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